

Alfalfa *Enod12* Genes Are Differentially Regulated during Nodule Development by Nod Factors and *Rhizobium* Invasion¹

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MsEnod12A and *MsEnod12B* are two early nodulin genes from alfalfa (*Medicago sativa*). Differential expression of these genes was demonstrated using a reverse transcription-polymerase chain reaction approach. *MsEnod12A* RNA was detected only in nodules and not in other plant tissues. In contrast, *MsEnod12B* transcripts were found in nodules and also at low levels in roots, flowers, stems, and leaves. *MsEnod12B* expression was enhanced in the root early after inoculation with the microsymbiont *Rhizobium meliloti* and after treatment with purified Nod factors, whereas *MsEnod12A* induction was detected only when developing nodules were visible. In situ hybridization showed that in nodules, *MsEnod12* expression occurred in the infection zone. In empty Fix⁺ nodules the *MsEnod12A* transcript level was much reduced, and in spontaneous nodules it was not detectable. These data indicate that *MsEnod12B* expression in roots is related to the action of Nod factors, whereas *MsEnod12A* expression is associated with the invasion process in nodules. Therefore, alfalfa possesses different mechanisms regulating *MsEnod12A* and *MsEnod12B* expression.

Early symbiotic interactions of leguminous plants and rhizobia comprise signal exchanges between the two partners. Nodulation of host plants requires bacterial factors and compounds identified through the extensive study of *Rhizobium* mutants. *Rhizobium* nodulation factors (Nod factors), produced after induction of *nod* genes by plant flavonoids, direct the initiation of nodule morphogenesis and infection through curled root hairs via infection threads (for review, see Hirsch, 1992). Nod factors of various *Rhizobium* species are lipooligosaccharides differing by modifications of a common oli-

gomer β -1,4-linked *N*-acetyl-D-glucosamine backbone. *Rhizobium meliloti* produces a sulfated tetramer containing a C₁₆ acyl chain with two double bonds, NodRm-IV(C16:2,S), that can be acetylated (Lerouge et al., 1990; Schultze et al., 1992). Purified Nod factors induce root hair deformations, preinfection thread formation, and cortical cell division (reviewed by Spalink, 1992). Furthermore, complex polysaccharides of the rhizobial outer surface are necessary for successful invasion of nodule cells, such as exopolysaccharides and lipopolysaccharides synthesized by the products of the *exo* and *lps* genes, respectively (reviewed by Gray and Rolfe, 1990).

The sequential expression of plant nodulin genes is associated with the onset of the early symbiotic events (Scheres et al., 1990b). Early nodulin genes, e.g. *Enod5* and *Enod12*, are induced during nodule development, whereas activation of late nodulin genes, e.g. the leghemoglobin genes, is correlated with nodule function (Nap and Bisseling, 1990; Scheres et al., 1990b). Analyzing early nodulin gene expression may contribute to the understanding of the regulatory pathways involved in nodulation.

Enod12, one of the most characterized early nodulin genes, was first isolated from a 21-d-old pea nodule cDNA library (Scheres et al., 1990a). The *Enod12* protein sequence is composed of a putative signal peptide followed by a stretch of Pro-rich repeats. It presumably represents a Hyp-rich glycoprotein of the cell wall. Two *Enod12* genes were identified in pea, *PsEnod12A* and *PsEnod12B*, that showed the same expression pattern (Govers et al., 1991). In situ hybridization of segments of inoculated pea roots and nodules of different ages revealed that *PsEnod12* might be involved in the infection process (Scheres et al., 1990a). Expression was found in cells containing infection threads and in cells preparing the passage for these structures. *PsEnod12A* and *PsEnod12B* were expressed in root hairs after inoculation with wild-type *Rhizobium leguminosarum* bv *viciae*, but not after infection with mutants defective in Nod factor synthesis (Scheres et al., 1990a). Recently, Horvath et al. (1993) demonstrated that in pea roots, purified rhizobial lipooligosaccharides induced the expression of both *Enod12* genes.

In *Medicago*, three *Enod12* genes have been identified as

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Abbreviation: RT, reverse transcription.

early nodulin genes, *MtEnod12* from the diploid *Medicago truncatula* (Pichon et al., 1992) and *MsEnod12A* and *MsEnod12B* from the tetraploid *Medicago sativa* (Allison et al., 1993). Like the *Enod12* gene from pea, *MtEnod12* is expressed in the infection zone of nodules. Induction as early as 3 to 6 h after infection with *R. meliloti* was shown for the *MtEnod12* promoter- β -glucuronidase fusion in transgenic *M. sativa* ssp. *varia* plants (Pichon et al., 1992).

We studied the expression pattern of the endogenous *M. sativa Enod12* genes. We found that *MsEnod12A* and *MsEnod12B* were differentially expressed not only in various plant tissues but also during the early symbiotic stages. We demonstrated that *MsEnod12B* was induced in roots treated with the cognate Nod factor, NodRm-IV(C16:2,S), whereas *MsEnod12A* was expressed in nodules being invaded by rhizobia. Our results indicate that in alfalfa, expression of the two *Enod12* genes are under different controls during symbiotic nodule development.

MATERIALS AND METHODS

Plant Material and Infection

For root harvesting, plants were grown and treated in the following ways. Alfalfa seeds (*Medicago sativa* ssp. *sativa* cv Sittel) were washed for 10 min in 95% ethanol and subsequently sterilized in 0.5% sodium dichloroisocyanurate (Bayrochlor, Bayrol, GMBH, Munich, Germany), 0.1% SDS (w/v) for 15 min. A row of 10 1-d germinated seedlings was placed on Petri plates containing nitrogen-free Gibson plant medium as described by Schultze et al. (1992). After 2 d in a growth chamber at 24°C under a 16-h light period, the roots were treated with *Rhizobium meliloti* or with purified Nod factors. For each treatment, about 15 to 20 plant roots were harvested.

R. meliloti was grown overnight in nitrogen-depleted basal medium supplemented with Glc and sodium succinate (GTS medium) (Kiss et al., 1979) in the presence of 1 μ M luteolin and resuspended at an A_{540} of 0.35 in a liquified 0.8% agarose solution containing 10 mM MgSO₄. This solution (15 μ L) was deposited in a spot on the root zone with growing root hairs. Wild-type *R. meliloti* strain 41 and the nonnodulating mutant derivative ZB138 (Kondorosi et al., 1984) were used. In this assay, small, white nodules were first visible 4 d postinoculation with strain 41. For harvesting, roots were dissected below the inoculation site and approximately 2 cm above the meristem of the main root.

For experiments with Nod factors, 15 μ L of liquid nitrogen-depleted Gibson medium, with or without 10⁻⁹ M purified NodRm-IV(C16:2,S) (Schultze et al., 1992), were distributed along the root. Action of Nod factor was verified 2 d later by root-hair deformation as described by Schultze et al. (1992). The action of modified Nod factor molecules was tested using Gibson medium supplemented with 10⁻⁹ M nonsulfated NodRm-IV(C16:2) (Truchet et al., 1991; Baev et al., 1992) and 10⁻⁹ M tetraacetyl chitotetraose (Sigma) in addition to 10⁻⁹ M NodRm-IV(C16:2,S) and the control without Nod factors.

For harvesting nodules and other plant tissues, alfalfa plants were grown under aeroponic conditions and treated as described by Allison et al. (1993). Similarly, mature spon-

taneous nodules were obtained in the aeroponic system on rooted shoot cuttings of alfalfa genotype A2 (NAR⁺ phenotype) after 5 weeks in the presence of low nitrogen-containing solution.

Empty nodules induced by Fix⁻ *R. meliloti* were collected as follows. One-day-old sterile alfalfa seedlings were transferred in pairs onto 15-mL nitrogen-depleted Gibson plant medium slants. Each plant was infected after 3 d with 75 μ L of liquid Gibson medium containing *R. meliloti* strain PP553 (Putnoky et al., 1990) at an A_{540} of 0.4. White Fix⁻ nodules of different ages were collected after 3 weeks.

RT-PCR

RNA was extracted using the guanidinium thiocyanate method and centrifugation through cesium chloride. RNA quality was checked on a formaldehyde gel (Sambrook et al., 1989). To avoid any genomic DNA contamination, 5 to 10 μ g of total RNA were treated for 30 min at 37°C with 10 units of RNase-free DNase I in a volume of 24 μ L in the presence of 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, and 12 units of RNAGuard (Pharmacia). After heat inactivation of the DNase, the RNA was precipitated with ethanol and resuspended in diethyl pyrocarbonate-treated water.

Multiple transcript analysis by RT-PCR was performed according to modified protocols of Sambrook et al. (1989) and Chelly et al. (1988). For greatest reproducibility, cDNA and PCR reaction samples were prepared from a single "master mix" of the appropriate reagents. Two to 5 μ g of DNase-treated RNA were reverse transcribed by 50 units of Moloney murine leukemia virus H⁻ Superscript reverse transcriptase (Gibco BRL) in a 30- μ L reaction mixture containing 100 pmol of oligo(pdT)₁₂₋₁₈, corresponding buffer (Gibco BRL), 10 mM DTT, 0.8 mM dNTP, and 14.4 units of RNAGuard (Pharmacia) during 1.5 h at 37°C.

Equal amounts of cDNAs (in general, one-tenth of the reaction mix) or 0.3 μ g of genomic DNA purified according to Dellaporta et al. (1983) were used for amplification in 100 μ L of corresponding buffer (Promega), 1.5 mM MgCl₂, 0.12 mM dNTP, 150 pmol of 5' and 3' *MsEnod12* primers (Allison et al., 1993), 10 pmol of *Rhe2* or *Msc27* primers (Allison et al., 1993), and 1.5 units of Taq polymerase (Promega). The *MsEnod12* primers described by Allison et al. (1993) were derived from the *MsEnod12A* sequence. The 24-bp long 3' *MsEnod12* primer differs in one nucleotide from the *MsEnod12B* sequence, namely at position four in front of the 3' end of this primer. Amplification from genomic *M. sativa* DNA showed that this single-nucleotide difference did not affect the amplification efficiency of the *MsEnod12B* fragment (see Fig. 1B, lane Ms). The sequence for the *Rhe2* 5' primer is 5'-CAGCCCATGATCAGCTCCC-3' and the sequence for the 3' primer is 5'-GAACCTGCTAGGCCAAGC-3'. Amplification was performed during 20 to 30 cycles of 1-min denaturation at 92°C, 1-min primer annealing at 55°C, and 1-min elongation at 72°C. RT-PCR was controlled by co-amplification of the endogenously expressed *Msc27* or *Rhe2*. *Msc27* (Györgyey et al., 1991; Pay et al., 1992; Allison et al., 1993; Csanadi et al., 1994) was similarly expressed in different tissues and nodules, as demonstrated by a northern blot with equal amounts of RNA loaded and also probed as

control to ribosomal DNA (our unpublished results). *Rhe2*, isolated from an alfalfa root hair cDNA library (L.A. Allison, unpublished results), was used as an internal control in root samples. By northern blot, no induction of this gene was detected after treatment with *R. meliloti* or Nod factors (L.A. Allison, unpublished results). *Rhe2* is homologous to genes from tobacco and *Arabidopsis* coding for channel proteins (Yamamoto et al., 1990). The exponential range of the PCR was tested by removing aliquots after various numbers of cycles from trial PCR reactions. After 20 cycles the amplification rate was in a linear range for all PCR products (data not shown).

Gel Electrophoresis and Southern Blot

According to Sambrook et al. (1989), one-tenth of the PCR products was separated on a 2% Tris-borate-EDTA agarose gel and transferred to a Hybond-N nylon membrane using a capillary blot system. The membrane was first hybridized to pBluescript containing an *MsEnod12B* 412-bp PCR fragment (see "In Situ Hybridization"). Due to the 96% sequence identity of the *MsEnod12A* and *MsEnod12B* PCR products, this probe revealed both bands. Following its removal, the blot was rehybridized to either an *Msc27* probe (Allison et al., 1993) or to *Rhe2* 234-bp PCR fragments. Probes were labeled with [³²P]dCTP.

In Situ Hybridization

A detailed description of the preparation of sections of 20-d-old nodules, fixation, and in situ hybridization to RNA probes was reported previously by Grosskopf et al. (1993). For generating the RNA probe, an *MsEnod12B* fragment was amplified with the *MsEnod12* 5' primer and a primer homologous to the 3' untranslated region of *MsEnod12B* (5'-CA-CTTGCCCTTGCCCAT-3') from a λEMBL4 genomic clone containing the *MsEnod12B* gene. The 412-bp PCR product was cloned into the *EcoRV* site of a pBluescript vector. The antisense RNA probe was obtained by in vitro transcription according to Grosskopf et al. (1993).

RESULTS

Differential Expression of *MsEnod12A* and *MsEnod12B*

MsEnod12A and *MsEnod12B* (Allison et al., 1993) and *MtEnod12* (Pichon et al., 1992) show high sequence homology among each other. Figure 1A shows the alignment of the encoded amino acid sequences. The major difference between the derived proteins is the length of the Pro-rich repeat region. *MsEnod12A* is the smallest protein with 11 Pro-rich repeats, each consisting of five amino acids, followed by *MtEnod12* and *MsEnod12B* with 13 and 15 repeats, respectively.

Due to the high 95% DNA sequence identity of *MsEnod12A* and *MsEnod12B* in the coding as well as in the 3' noncoding region (Allison et al., 1993), we could not follow the expression of the two genes separately by any conventional hybridization technique. Therefore, the expression pattern was investigated by applying an RT-PCR method, using oligonucleotides flanking the deletion in the Pro-rich repeat region

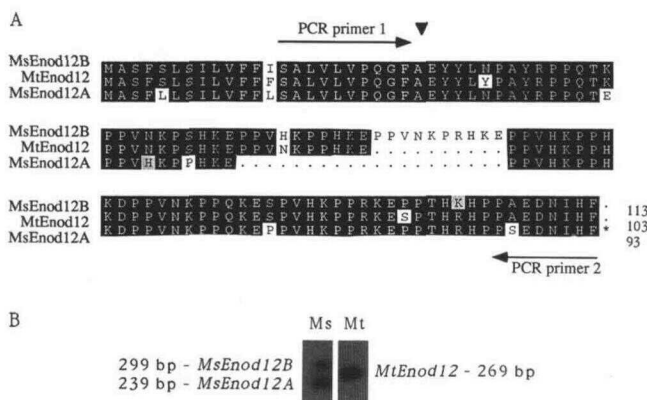


Figure 1. Comparison of the *Enod12* sequences in *Medicago*. A, Alignment of the amino acid sequences of *MsEnod12B*, *MtEnod12*, and *MsEnod12A*. Black outlining indicates identical amino acids, gray outlining shows conservative substitutions. Dots within the sequence show deletions in the Pro-rich repeat region. The arrowhead marks the end of the putative signal peptide. The positions of *MsEnod12* primers 1 and 2 are indicated by arrows. B, PCR analysis of *Enod12* genes in *M. sativa* and *M. truncatula*. *Enod12* sequences were amplified during 30 cycles from 0.3 µg of genomic DNA, separated by electrophoresis, and transferred to a membrane that was hybridized to an *MsEnod12B* probe. Lanes: Ms, *M. sativa* ssp. *sativa* cv Nagyszénási; Mt, *M. truncatula* PCR products with indicated sizes.

as indicated in Figure 1A. The PCR DNA fragments of *MsEnod12A* and *MsEnod12B* differ in size by 60 bp as shown in Figure 1B. After amplification of genomic *M. sativa* DNA, two PCR bands were visible of the expected sizes of *MsEnod12A* and *MsEnod12B*, namely 239 and 299 bp (Fig. 1B, lane Ms). Genomic DNA from *M. truncatula* gave a single intermediate PCR band corresponding to the expected 269-bp size (Fig. 1B, lane Mt; Pichon et al., 1992).

We established an approach for multiple transcript analysis that proved to be the most reliable for our experiments. DNase-treated total RNAs from different plant tissue samples were reverse transcribed with an oligo(dT) primer. From these cDNA mixtures, *Enod12* and an endogenously expressed control gene, *Msc27* or *Rhe2*, were simultaneously co-amplified with the appropriate oligonucleotide primer pairs in the same reaction tube. The intensity of the DNA fragment of the control gene after PCR reflected sample-to-sample variations in RT and PCR, and monitored the eventual extent of RNA degradation during the manipulations. We chose controls generating single products after amplification. The constitutively expressed *Msc27* (Györgyey et al., 1991; Pay et al., 1992; Allison et al., 1993; Csanadi et al., 1994) was used as an internal control in different tissue and nodule samples. *Rhe2* (L.A. Allison, unpublished results), a gene constitutively expressed in roots, was used to control the RT-PCRs in root samples (see "Materials and Methods"). For quantitation, the ratios between control and *Enod12* amplification fragments had to be comparable (for a recent review about quantitative RT-PCR, see Foley et al., 1993). In our case, the control transcripts were more abundant than the *Enod12* sequences. To overcome this problem and a possible effect of out-

titration of the low-abundant *Enod12* sequences (described by Murphy et al., 1990), the primer concentrations were reduced for the controls (down to 10% of the optimum concentration) and increased for *Enod12* (150% of the optimum concentration). Analyzing trial PCRs after various numbers of cycles demonstrated that control and *Enod12* amplifications were in the linear range after 20 to 25 cycles (data not shown).

The expression of *MsEnod12A* and *MsEnod12B* was studied in various plant tissues. *MsEnod12A* was expressed in nodules but it was not detected in any other tissue as seen in Figure 2. In contrast, *MsEnod12B* transcripts were found in nodules and low amounts were also detected in roots, flowers, stems, and leaves (Fig. 2). In 5-d-old seedling roots (Fig. 2, lane r), *MsEnod12B* was more expressed than in the roots of 4-week-old nodulated plants. The expression was analyzed in the middle and lower parts of these roots after excision of nodules (Fig. 2, lanes rm and rl). We excluded the possibility that the faint *MsEnod12B* PCR bands were due to contaminating genomic DNA fragments because no *MsEnod12A* signals were found (compare to Fig. 1B, lane Ms). These results indicated that *MsEnod12A* and *MsEnod12B* were differentially expressed in various plant tissues.

In a further experiment, the induction of the two genes was followed during nodule development. Three-day-old alfalfa seedlings were inoculated with the wild-type strain *R. meliloti* Rm41, and as a control, with the nodulation-deficient mutant ZB138. After 2, 4, and 6 d, the regions of the roots below the spot-inoculation site were dissected for transcript analysis. Figure 3 shows that a low amount of *MsEnod12B* transcripts was found in all root samples. After inoculation with Rm41, *MsEnod12B* expression increased until d 6, whereas after inoculation with the nodulation-deficient mutant, it stayed at a constant, low level during this same time period. Expression of *MsEnod12A* was detectable only 4 and 6 d after inoculation with Rm41, when nodules first became visible (Fig. 3). The control for RT-PCR was *Rhe2* amplifica-

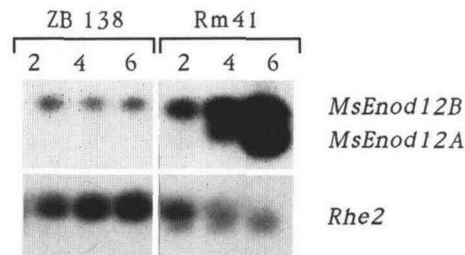


Figure 3. RT-PCR analysis of *MsEnod12A* and *MsEnod12B* induction during nodule development. RNA from root samples harvested at different time points after *R. meliloti* inoculation was used for RT and co-amplification during 20 cycles of *MsEnod12* and *Rhe2* sequences. The PCR products were hybridized subsequently to an *MsEnod12B* and an *Rhe2* probe. The first three lanes (ZB138) represent control roots harvested at several time points after ZB138 inoculation, namely after 2, 4, and 6 d; the next three lanes (Rm41) show roots collected 2, 4, and 6 d after Rm41 infection.

tion. Since only Rm41 but not ZB138 is capable of producing Nod factors, these results indicated that the lipooligosaccharide molecules might be signals inducing *MsEnod12* gene expression.

Effect of Nodulation Factors on *MsEnod12A* and *MsEnod12B* Expression

The effect of purified Nod factors on *MsEnod12A* and *MsEnod12B* gene expression was tested. Three-day-old plant seedlings were treated with the cognate Nod factor NodRm-IV(C16:2,S) at a concentration of 10^{-9} M for different time periods. As a control, plants were mock-inoculated with plant medium. Roots were cut into three zones: zone 1, next to the meristem and devoid of root hairs; zone 2, containing growing root hairs; and zone 3, with mature root hairs, as shown in Figure 4C. Expression of *MsEnod12B* was enhanced in the root hair zones 2 and 3 after Nod factor treatment (Fig. 4A). The highest *MsEnod12B* transcript level in zone 2 was detected 6 h after treatment, and in zone 3 1 d after treatment. In the meristematic region, *MsEnod12B* expression was not enhanced. Root-hair deformations were observed after 1 d in zones 2 and 3. At 2 and 3 d after Nod factor application, the *MsEnod12B* transcript level was not significantly different from the control in any zone (data not shown). Minor differences in the control *Rhe2* amplification were not sufficient to account for the relatively large differences observed for *MsEnod12B*, indicating comparable inputs of cDNA quantities and PCR efficiencies in the different samples (Fig. 4A). No major differences in the level of the *MsEnod12B* transcripts were found if Nod factor concentrations from 10^{-13} M to 10^{-7} M were used. Nod factor below a concentration of 10^{-13} M did not enhance *MsEnod12B* expression (data not shown). After a longer exposure of the filter hybridization of Figure 4A, *MsEnod12B* signals were visible in all root samples (not shown) except *MsEnod12A*. At the different concentration tested, the cognate Nod factor was not able to elicit *MsEnod12A* expression in roots in any of the tested times (up to 4 d).

In further experiments, unsulfated Nod factor NodRm-

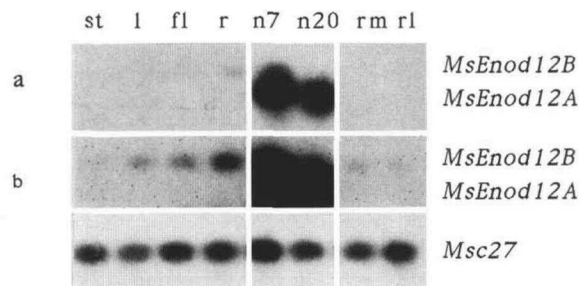


Figure 2. RT-PCR analysis of *MsEnod12A* and *MsEnod12B* expression in different plant tissues. *Enod12* and the control *Msc27* sequences were co-amplified during 25 cycles from RNA of various plant tissues of *M. sativa* ssp. *sativa*. After electrophoresis and transfer blot, they were subsequently hybridized to an *MsEnod12B* or *Msc27* probe. The different lanes contain RT-PCR products from stems (st) and leaves (l) both from 9-d-old plants; flowers (fl); untreated roots of 5-d-old seedlings (r); 7- (n7) and 20-d-old (n20) nodules; middle (rm) and lower (rl) root parts of 4-week-old nodulated plants after excision of nodules. A short (a) and a long (b) exposure of the *MsEnod12B* hybridization filter are shown.

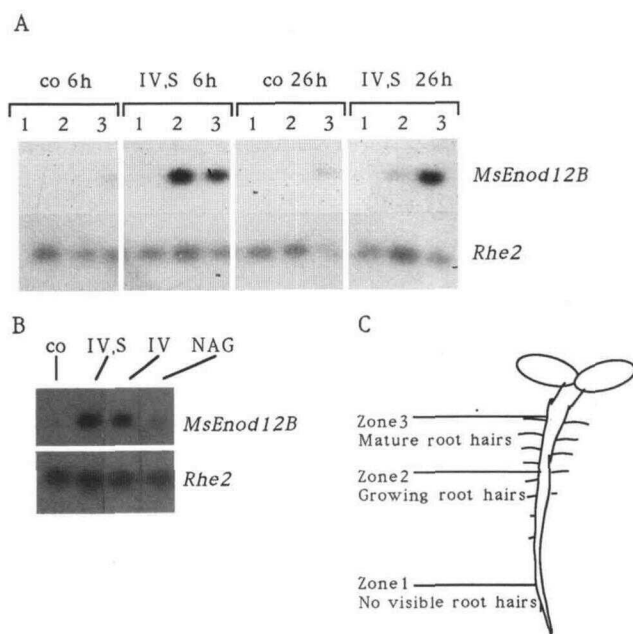


Figure 4. RT-PCR analysis of *MsEnod12B* induction after treatment with Nod factors. A, RT-PCR was performed on roots treated with 10^{-9} M NodRm-IV(C16:2,S) for 6 and 26 h and on mock-inoculated control roots cut into three zones as shown in C. The co-amplified *MsEnod12* and *Rhe2* fragments (20 PCR cycles) were subsequently hybridized to an *MsEnod12B* and an *Rhe2* probe. The first six lanes contain the root samples after mock-inoculation (co 6h) and treatment with Nod factor for 6 h (IV,S 6h) harvested from zones 1, 2, and 3; the next six lanes represent control roots (co 26h) and Nod factor-treated roots after 26 h (IV,S 26h). 1, 2, and 3 indicate the root zones from which RNA was prepared. B, Roots were treated with 10^{-9} M modified Nod factors for 6 h. Root pieces of zone 2 were dissected and used for RT-PCR analysis as described above. The different lanes represent the PCR products of mock-inoculated control roots (co); NodRm-IV,S (IV,S); NodRm-IV (IV); and chitotetraose-treated roots (NAG). C, Schematic representation of the root of an alfalfa seedling. Zone 1 corresponds to the meristematic zone, zone 2 is the zone with growing root hairs, and zone 3 is the zone with mature root hairs.

IV(C16:2) and *N*-acetyl glucosamine tetrasaccharide were tested for their capacity to enhance the expression of the *MsEnod12B* gene, both at the concentration of 10^{-9} M. After a treatment of 6 h, root pieces of zone 2 were harvested. Nonsulfated Nod factor with reduced abilities for root-hair deformation (Roche et al., 1992) significantly enhanced *MsEnod12B* expression (Fig. 4B, lane IV). After treatment with chitotetraose, no enhancement of *MsEnod12B* expression was found (Fig. 4B, lane NAG). Again, *MsEnod12A* transcripts were not detectable in roots. These results indicate that expression of *MsEnod12B* but not *MsEnod12A* can be modulated by Nod factors in the root.

Expression of *MsEnod12A* and *MsEnod12B* Is Associated with Infection

The site of *MsEnod12* gene expression in nodules was localized by in situ hybridization of a 20-d-old *M. sativa* ssp.

sativa nodule using a *MsEnod12B* probe (Fig. 5). Hybridization signals indicating *MsEnod12* expression occurred in cells of the infection zone near the nodule meristem where the plant cells are invaded through infection threads. In addition, hybridization signals were observed in the peripheral tissue of the nodule. These additional signals might be explained by cross-hybridizing RNA coding for a Pro-rich protein. A cross-hybridizing transcript has been seen on a northern blot containing nodule RNAs probed against *MsEnod12* sequences (Allison et al., 1993). To study whether *Enod12* induction is related to the process of infection, we examined its expression by RT-PCR in nodules blocked in the invasion step. Empty nodules were obtained by inoculation with the *Fix*⁻ *R. meliloti* strain PP553 (Putnoky et al., 1990) mutated in the *exoB* and *fix-23* genes and consequently lacking outer surface exopolysaccharides and capsular polysaccharides required for invasion. In nodules induced by this strain, infection threads are aborted and only very few cells are invaded (Putnoky et al., 1990). *Fix*⁻ nodules of different developmental stages were harvested 3 weeks after inoculation. *MsEnod12A* was expressed at a much lower level in these empty nodules than in 7-d-old white or 19-d-old nitrogen-fixing wild-type nod-

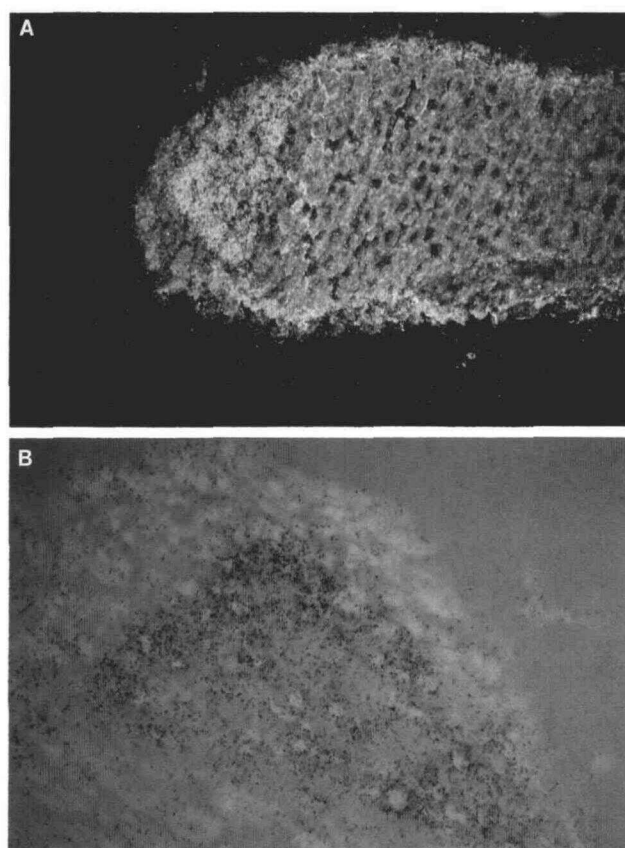


Figure 5. Localization of *MsEnod12* expression in mature alfalfa nodules. In situ hybridization was performed on sections of a 20-d-old *M. sativa* nodule. A ³⁵S-labeled *MsEnod12B* antisense probe was used. The infection zone showed a high density of silver grains in this section. B shows a higher magnification of the infection zone than A.

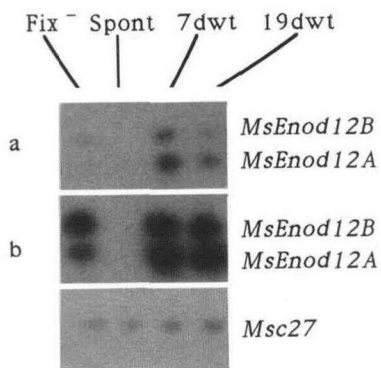


Figure 6. RT-PCR analysis of *MsEnod12A* and *MsEnod12B* expression in *Fix*⁻ and spontaneous nodules. *MsEnod12* and *Msc27* sequences were co-amplified from various nodule samples. After gel electrophoresis they were transferred to a membrane and hybridized subsequently to an *MsEnod12B* and an *Msc27* probe. The lanes show amplification products from *Fix*⁻ nodules (*Fix*⁻); mature spontaneous *M. sativa* ssp. *varia* nodules (*Spont*); 7- and 19-d-old wild-type nodules (7dwt and 19dwt, respectively). a and b show the *MsEnod12A* and *MsEnod12B* amplification after 20 and 25 PCR cycles, respectively. The *Msc27* signals were obtained after 20 cycles.

ules (Fig. 6, lanes *Fix*⁻, 7dwt, and 19dwt). The *MsEnod12B* transcript level in *Fix*⁻ nodules was comparable to the one in 19-d-old wild-type and slightly lower than in 7-d-old wild-type nodules (Fig. 6). Reduced expression in *Fix*⁻ nodules compared to wild-type nodules was more striking for *MsEnod12A* than for *MsEnod12B*. We also analyzed the expression in spontaneous nodules, obtained after nitrogen starvation on certain genotypes of *M. sativa* in the absence of bacteria (Truchet et al., 1989). In mature spontaneous nodules of *M. sativa* ssp. *varia* genotype A2, neither of the two transcripts was detectable (Fig. 6, lane *Spont*). Both *MsEnod12* genes can be amplified readily from genomic DNA of the subspecies *varia* (not shown). The control *Msc27* bands were amplified in all cases, which proved that in the samples analyzed cDNA synthesis and PCR had taken place correctly (Fig. 6). These results indicate that *MsEnod12A* expression correlates with the presence of rhizobia inside nodules.

DISCUSSION

We demonstrated here that the two early nodulin genes *MsEnod12A* and *MsEnod12B* are differentially expressed during symbiosis, providing evidence for distinct mechanisms regulating these two genes in alfalfa. Our results showed that *R. meliloti* Nod factors induce the expression of only *MsEnod12B*. Hence, *MsEnod12A* exhibits a novel *Enod12* expression pattern, different from all other *Enod12* genes, since it is not induced by *Rhizobium* lipooligosaccharides. *MsEnod12A* expression is related to the invasion process in nodules.

After infection with *R. meliloti*, *MsEnod12B* was rapidly induced in roots, prior to induction of *MsEnod12A*. This *MsEnod12B* expression increased until nodules became visible. This early induction probably occurred in the epidermis and in root hairs as demonstrated for the *Enod12* genes in

pea (Scheres et al., 1990a) and *M. truncatula* roots (Pichon et al., 1992). Accordingly, an enhancement of *MsEnod12B* transcription in roots was detected after treatment with the cognate Nod factor at the concentration of 10^{-9} M. This induction took place in the root zones susceptible for root-hair deformations. However, after application of the Nod factor, the effect occurred transiently, reaching a maximum level 6 to 26 h after treatment. The transient effect indicates that the continuous presence of Nod factors might be necessary for a prolonged *MsEnod12B* expression, as is the case during contact with rhizobia. Degradation of Nod factors due to plant chitinases (Staehelin et al., 1994) could be an explanation for this transient expression. *MsEnod12B* was induced by the sulfated and the nonsulfated NodRm-IV(C16:2), whereas the unsubstituted sugar backbone alone was not able to induce the gene. Recent studies by other groups also indicate that *Enod12* genes can be induced by noncognate Nod factors. Horvath et al. (1993) demonstrated that besides *R. leguminosarum* bv *viciae* NodRlv metabolites, lipooligosaccharides from *R. meliloti* were able to trigger the expression of the *PsEnod12* genes in pea. Pichon et al. (1993) reported induction of *MtEnod12* by nonsulfated Nod factor at concentrations of 10^{-9} M and higher. In this case, sulfated NodRm-IV(C16:2,S) was active at a concentration range 4 orders of magnitude lower, indicating that a specific structure was required for *MtEnod12* induction by low Nod-factor concentrations.

MsEnod12B was expressed at a low level in various plant organs, and therefore is not a true early nodulin gene, according to the definition by van Kammen (1984). Expression in nonsymbiotic tissues was also demonstrated for *PsEnod12A* and *PsEnod12B*, specifically in flowers and stems (Scheres et al., 1990a; Govers et al., 1991). In contrast to the pea *Enod12* genes and to *MtEnod12*, *MsEnod12B* was expressed at a basal level in untreated roots. The expression was higher in 3-d-old seedling roots than in roots of plants grown for more than a month (Fig. 2). A similar down-regulation of *MsEnod12B* expression was observed during the aging of wild-type nodules (Allison et al., 1993). The very low level of expression in mature tissues could also explain the failure to detect *MsEnod12B* signals in mature spontaneous nodules. During formation of these spontaneous nodules, a higher expression level might have occurred.

MsEnod12A was induced at a later stage during nodule development than *MsEnod12B*, and could not be induced in roots after application of Nod factors. Therefore, we conclude that in root cells, *MsEnod12A* cannot be regulated by *Rhizobium* lipooligosaccharides, as would be the case for all other *Enod12* genes characterized (Horvath et al., 1993; Pichon et al., 1993). *MsEnod12A* expression was detectable only 4 d after infection with *R. meliloti* when nodules first became visible. Presumably, the transcription started in nodule primordia, where it increased rapidly to a high level during nodule development. In situ hybridization of a nitrogen-fixing nodule with a *MsEnod12* probe showed hybridization signals in the infection zone and in the periphery of the nodule but not in the central symbiotic zone, providing evidence for the role of *MsEnod12* genes in the infection process. However, due to the high homology of the two *MsEnod12* genes, precise localization of the expression of each gene could not be addressed by this technique. In empty

nodules, *MsEnod12A* expression was much reduced compared to wild-type nodules, correlating with the low frequency of invasion observed in nodules induced by *exoB* and *fix-23* double mutants (Putnoky et al., 1988). The low level of *MsEnod12A* expression in empty nodules and the absence of *MsEnod12A* transcripts in spontaneous nodules suggest that *MsEnod12A* expression correlates with *R. meliloti* invasion. Moreover, *MsEnod12A* is a true early nodulin gene (van Kammen, 1984), with activation occurring only in nodules but not in roots or any other plant organs.

MsEnod12A and *MsEnod12B* show novel expression patterns compared to the *Enod12* genes of pea or *M. truncatula*. *MsEnod12B* expression takes place in a way similar to the induction of *MtEnod12* during symbiosis. Most information about the *M. truncatula* *Enod12* gene expression, however, is derived from the behavior of a *MtEnod12* promoter- β -glucuronidase fusion in a *M. sativa* background. Further evidence for a close relationship of *MsEnod12B* with *MtEnod12* was recently provided by Csanadi et al. (1994), who traced the evolution of *Medicago* species based on *Enod12* sequences. They calculated that after divergence of *Medicago* species, *MsEnod12A* as well as *MtEnod12* would have evolved independently through deletions from an *MsEnod12B*-type gene present in a common ancestor plant.

In conclusion, early nodulin gene expression patterns differ among legume species. Alfalfa is interesting in the analysis of *Enod12* gene transcriptional control, since different regulatory pathways may control the activation of *MsEnod12A* and *MsEnod12B* during symbiosis. Transgenic *M. sativa* ssp. *varia* plants containing promoter- β -glucuronidase fusions for both *MsEnod12* genes are being constructed in our laboratory to study the signals required for *MsEnod12A* and *MsEnod12B* expression.

Tissue- and cell-specific expression patterns were also found for members of other Hyp-rich glycoprotein gene families whose encoded proteins differ primarily in the composition and the number of Pro-rich repeats (see, for example, Hong et al., 1989; Wyatt et al., 1992). The importance of the organization of the Pro-rich repeat region for the properties of Hyp-rich glycoproteins is still unknown. We speculate that *MsEnod12A* and *MsEnod12B* might be involved in distinct cell-wall structures of root hairs, roots, and nodules arising during the infection process leading to symbiosis.

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